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Molecular plasticity of retinal ganglion cells after partial optic nerve injury

Michael R. Kreutz[1,2], Constanze I. Seidenbecher[1] and Bernhard A. Sabel[1]

[1]*AG Mol. and Cell. Neurobiology; Institute of Medical Psychology; Otto-von-Guericke University, Magdeburg, Germany*
[2]*Department of Neurochemistry/Molecular Biology; Leibniz Institute for Neurobiology; Magdeburg, Germany*

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Abstract

In the past few years we established the partial crush of the optic nerve as an *in vivo* model system for the study of signaling pathways involved in molecular plasticity after axonal injury. The simplicity of this model at the cellular level allows decisive questions to be answered whilst functional aspects of visual information processing can be studied in parallel. A major advantage of a partial optic nerve crush model is the opportunity to directly compare different cell populations: (i) the rapidly degenerating retinal ganglion cells (RGC), (ii) the axotomized RGC population that eventually dies over the period of the next few weeks, (iii) the axotomized RGC population surviving for a long time in the retina without an axon and (iv) the surviving RGC population that maintains axonal connections to their brain targets. Thus, differential aspects of post-lesion plasticity between axotomized and non-axotomized cells can be studied and gene transcription leading either to cell death or survival can be analyzed. Using this axonal injury model we investigated the expression of immediate early genes, glutamate receptors, and other differentially expressed genes that we identified with a combined subtractive hybridization and suppression polymerase chain reaction (PCR) screen. Moreover, we characterized time course of cell death, the astroglia response of the retina and optic nerve as well as the topography of anterograde and retrograde axonal transport.

Keywords: axonal lesions, cell death, glutamate receptors, signaling pathways, gene transcription, c-jun, adhesion kinases

1. Introduction

Traumatic brain injury is characterized by the occurrence of diffuse axon injury throughout the brain that exacerbates the neuropsychological deficits of trauma patients [17]. Axon stretch, compression, or destruction leads to disconnection of brain nuclei and especially long-projecting fibers, which, when compromised in such a manner, may affect widely distributed areas throughout the brain [37]. The extent of diffuse axonal injury correlates in primates with the severity of the traumatic head injury and with the resulting severity and duration of coma [16], a situation that has also been described in man [17]. In addition, secondary neurodegeneration after closed head neurotrauma is thought to be partially mediated by mechanical shear stress of axons, which is induced by stretch of the white matter. Mechanical injury leads to axon shear, stretch, and rupture, resulting either in secondary axotomy or internal axon damage. In the case of axotomy, structural and functional loss are the consequence. Here, repair and functional restitution is only possible by regrowth of axons to their target (regeneration). In the case of internal axonal injury, where the axons are still in contact with their targets, axonal repair is conceivable without the need of regeneration.

* Correspondence to: Prof. B. A. Sabel, Inst. for Medical Psychology, Medical Faculty, Otto-von-Guericke University, Leipziger Str. 44, D - 39120 Magdeburg, Germany; Tel.: +49-391-611-7100; Fax: +49-391-611-7103; E-mail: Bernhard.Sabel@medizin.uni-magdeburg.de

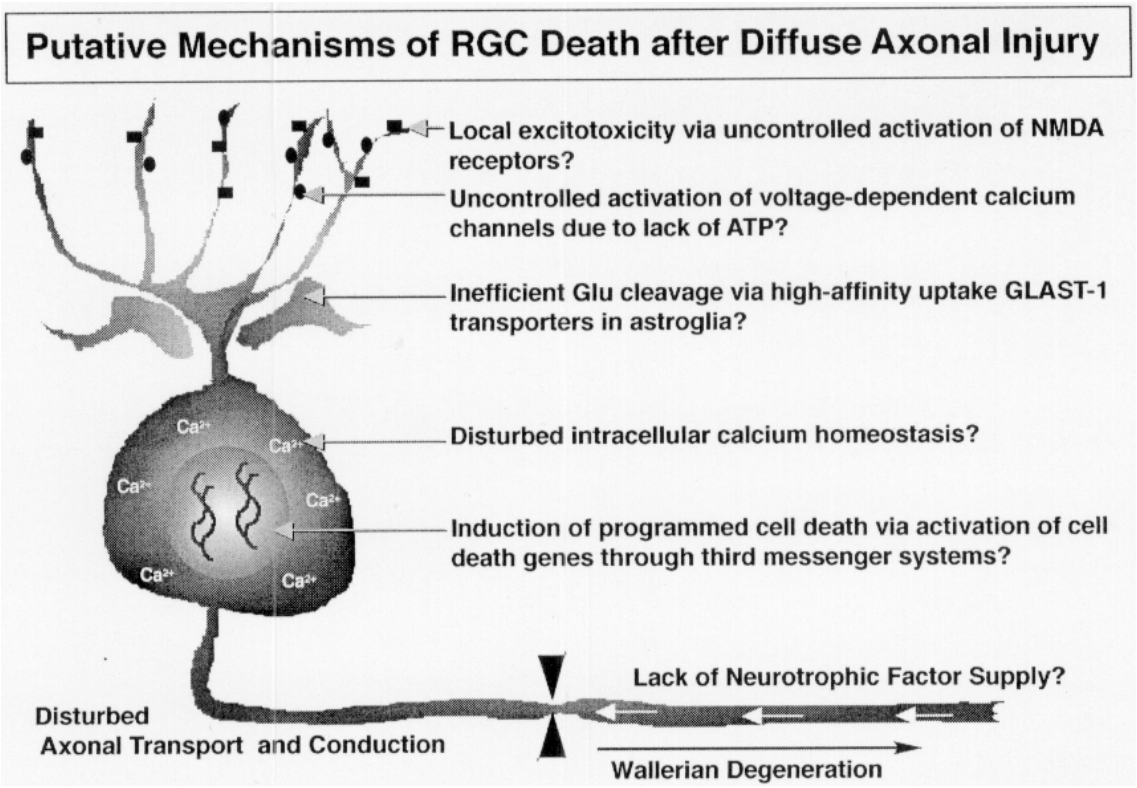

Fig. 1. Optic nerve crush leads to massive degeneration of RGC. The cellular mechanisms responsible for RGC death, however, are not yet completely understood. An axonal dying back mechanism [60,61], blockade of axonal transport and a shortage of neurotrophic factor supply [7,47] (see also [14,34]), activation of signaling pathways leading to apoptosis [26], uncontrolled activation of voltage-dependet Ca^{2+} channels [8, 55] and altered intracellular Ca^{2+} homeostasis [42,50] as well as local excitotoxicity [39,65] have all been implicated in the degenerative response to axonal injury. At present it is unclear how these different mechanisms interact and which signaling pathway from the injured axon to the cell soma and nucleus initially triggers the cellular response to the insult.

2. Partial optic nerve injury as a model of diffuse axonal injury

Controlled optic nerve crush has been suggested as a model for diffuse axonal injury [13,41]. In contrast to axotomy, two populations of retinal ganglion cells (RGCs) survive crush injury in this lesion model, i.e. a population which is still connected to the optic tectum (10–15 % of the entire poulation) two weeks postinjury [43] and a not yet quantitatively determined number of axotomized RGC which also reside in the retinal ganglion cell layer devoid of a projecting axon [30]. This has also been reported after a complete crush of the nerve [9,35]. Optic nerve crush has been found to affect the expression of a variety of gene products, including proteins coupled to Ca^{2+}-dependent second messenger pathways [28] and growth factors [62]. However, the mechanisms of cell death are not yet well understood (see also Fig. 1).

To date, our knowledge about signaling pathways from the injured axonal compartment to the cell nucleus is limited. It is therefore important to identify mechanisms of retrograde axonal signaling that are crucially involved in the regulation of gene transcription that either leads to cell survival or cell death. The identification of such mechanisms could pave the way for future pharmacological interventions that will promote the clinical outcome of both, diffuse axonal injury and axotomy after neurotrauma.

The scope of our approach therefore was first to determine how and by which mechanisms cellular degeneration occurs. Then we sought to identify how axonal injury alters RGC gene expression. In a first set of experiments, we studied the question of which trancription factors could be responsible for the early transcriptional regulation in response to nerve crush. In a second set of experiments we were interested in understanding whether or not axonal injury alters the expression of gluatamate receptor genes of RGC and which role this could play in postlesion plasticity. Finally, we analyzed the retino-fugal projections and tried to identify signaling pathways from the injured axonal compartment to the nucleus.

3. Cellular degeneration of RGC in the retina after partial crush injury

Cell death in the retinal ganglion cell layer occurs in many disease states such as glaucoma, ischemia or mechanical trauma of the optic nerve. It has been suggested that in these pathological states excitotoxicity is the final common pathway leading to cell death [33] and RGC degeneration after optic nerve injury is at least in part mediated by the un-

controlled activation of NMDA receptors [39,65]. At the morphological level, different types of retinal cell death have been described in response to injury, but it is still unclear as to what extent cell death is associated with necrotic and apoptotic cellular profiles. At least axotomy of the optic nerve was demonstrated to be followed by apoptotic cell death [3,26,38].

We have recently investigated the time course of cellular degeneration in the partial crush model [4]. *In situ* end-labeling of DNA fragments with the TUNEL method revealed first apoptotic cellular profiles 5 days postcrush. Massive apoptotic cell death and pyknotic cell bodies were found 1 week post-injury. A considerable number of TUNEL-labeled cells was also found after 2 and 4 weeks postinjury. However, *in vitro* retinal whole mount preparations revealed that during postlesion day 2–5, many cell bodies with ruptured membranes as evidenced by nucleosomal Sytox-staining were present. Moreover, at early stages of RGC degeneration necrotic cellular profiles were also found. Thus, both necrosis and apoptosis follow in sequelae partial optic nerve injury with a distinct boundary in their occurance over time.

As already outlined above, the causes of RGC death after axonal injury are currently not well understood. Silveira and coworkers [48] suggested that the rapid phase of RGC death between day 3–7 after axotomy is caused by an overactivation of glutamate receptors in a subset of RGC which results in local excitotoxicity. It has also been recently shown that NMDA receptor antagonists effectively attenuate RGC death after optic nerve crush [39,65]. These data suggest that at least a significant proportion of RGC degenerate by a final common pathway involving NMDA receptor activation. The question arises as to whether the conceivable different causes of cellular degeneration in the retina result in either necrosis or apoptosis and if severity of the axonal lesion determines whether necrotic or apoptotic cell death occurs. Supporting evidence for a continuum of necrotic and apoptotic cellular profiles depending on the severity of the insult has been found under conditions of excitotoxicity [1,6].

4. The astroglia response to partial crush injury in the retina

Cellular degeneration in the brain is accompanied by a regulated response of neuroglia to the insult (for review see [32]). Although astroglia proliferation as a consequence of mechanical retinal injury has been investigated in detail (see for instance [24,63]), only few reports exist on the activation of macroglia after optic nerve axotomy or transection [25,45]. In these reports a transient increase of GFAP-immunoreactivity in Müller glia but not in astrocytes was found. We characterized the time course and occurence of GFAP-immunopositive reactive astroglia in the retina in response to partial optic nerve crush. Surprisingly, we found no increase in GFAP-staining in retinal cryostat sections after a partial crush of the optic nerve over a time course of two weeks (Engelmann and Kreutz, unpublished observations). Local retinal lesions induced by xenon-irradiation, however, were followed by a significant increase in GFAP-immunoreactivity in the injured area, demonstrating that the used staining method was sensitive to detect astroglia proliferation. Moreover, western blot analysis confirmed that GFAP-immunoreactivity is not increased in retinal protein homogenates after crush, whereas increased GFAP-immunoreactivity was found in optic nerve protein preparations 1 week after crush. This increase was confined to the crush site. Systemic administration of the inflammatory agent lipopolysaccharide (LPS) alone had also no effect on GFAP-immunopositive cells in the retina and the optic nerve. Interestingly, a combination of optic nerve crush with a parallel administration of LPS leads to a significant increase of GFAP-immunoreactivity in retinal cryostat sections as judged by immunostainings. These data demonstrate that despite a cell loss of about 80 % of all RGC after optic nerve crush increased GFAP-immunopositive astroglia is only found in close proximity to the lesion site but not in the retina. Moreover, a parallel LPS-induced inflammatory response is necessary to induce GFAP-positive reactive astrogliosis in the retina after partial optic nerve injury.

5. Glutamate receptor expression in response to optic nerve injury

Glutamate receptors are known to play a crucial role in neuronal cell death in a variety of disease states [33]. However, little is known, about the consequences of neuronal injury on glutamate receptor gene expression. It has been shown that NMDA receptor antagonists have potent neuroprotective effects after axotomy of the optic nerve [38,65], thereby indicating that activation of NMDA-receptors is involved in cellular degeneration. Hence, morphological evidence suggests alterations of the dendritic tree and soma size of axotomized RGCs [51], which is also evidence in favour of a reorganization of the postsynaptic membrane of injured RGC. A provocative hypothesis is therefore that axonal injury might lead to a reorganization of synaptic contacts and the molecular make-up of glutamate receptors in the dendrite of the affected cell population. We have taken advantage of the easy accessibility of the optic nerve and used the crush model to investigate whether axonal injury alters the expression of NMDA- and AMPA-receptor genes in the retinal ganglion cell layer [30] thereby possibly altering the excitability of RGC.

The cellular specific splicing of the retinal NMDAR1 receptor (NR1) and expression of NMDAR2 (NR2) subunits in response to optic nerve injury was investigated by *in situ* hybridization in adult rats. Transcript levels for the NR2a-c subunits were clearly reduced after the lesion. Moreover, a controlled optic nerve crush led to a clear-cut alteration in the expression of alternatively spliced NR1 variants in the retinal ganglion cell layer with the preferential expression of the NR1-2b and NR1-4b isoforms especially between two days and one week post-injury. The cellular label for all oth-

er isoforms remained unchanged or was clearly attenuated and steadily downregulated to barely detectable levels within 4 weeks. To directly test the hypothesis that NR1-b expression is crucial for cell survival after axonal trauma, we intraocularly administered an antisense oligonucleotide against the NR1-b isoform 2 and 3 days after injury. This led to a drastic loss of retrogradely labeled RGCs. These findings point towards trauma-specific changes in alternative splicing of the NR1 receptor which are crucial for the survival of RGC after partial axonal injury. NR1 receptors lacking the N-terminal insert encoding NR1-b are more sensitive to proton inhibition [54] and are selectively potentiated by micromolar concentrations of [Zn^{2+}] [21] or polyamines at saturating glycine concentrations [11,12]. Splice variants with C-terminal deletions (NR1-2 and NR1-4) show no differences in their basic responses, but exhibit less susceptibility to PKC phosphorylation [53]. Thus, optic nerve injury seems to induce NR1 isoforms with fewer phosphorylation sites, which are blocked by extracellular [Zn^{2+}] and have a decreased susceptibility to proton inhibition. We propose that these altered splicing events reflect an adaptive response of cells in the ganglion cell layer to a changed environment after trauma. It has been shown that brain injury decreases the pH of the extracellular fluid [57] and may thereby increase the proton inhibition of the NR-1 [54]. The altered splicing could lead to a different composition of the native NMDA receptor and different responses to glutamate activation. It is very likely that this reflects an adaptive and not a pathogenic cellular response to a changed environment.

AMPA receptors consist of hetero-oligomers composed of four subunits, GluR1-4. Expression of GluR2 determines the low Ca^{2+}-permeability of native AMPA-receptors. *In situ* hybridization experiments with retinal cryostat sections revealed a significant downregulation of GluR2 transcripts in the retinal ganglion cell layer in response to optic nerve injury. Significantly lower hybridization signals were found as early as two days after crush, indicating that the attenuated expression is not a consequence of RGC cell loss. One week post-injury RGC retrogradely labeled with fluorogold do not express detectable GluR2 transcripts. Accordingly, GluR2 protein levels were reduced one week post-injury as judged by western blot analysis of retinal protein homogenates. The downregulation of GluR2 transcripts was still observed 4 weeks after optic nerve crush, showing that the regulation of receptor expression is not transient. No significant alteration was observed in the expression of GluR1, GluR3 and GluR4 subunits in the ganglion cell layer at the cellular level. Thus, transcript levels of these AMPA-receptor subunits remained largely unchanged in the surviving cell population. We hypothesize that axonal injury leads to an altered excitability of surviving RGCs. Moreover, AMPA receptors in the surviving population could exhibit an increased Ca^{2+}-permeability.

In summary, our data on the expression of glutamate receptor genes after crush injury suggest that axonal injury probably changes the excitability of the surviving RGC population. Thus, it is tempting to speculate that after an axonal lesion neurons are capable of altering the molecular make-up of the postsynaptic membrane and thereby can alter their dendritic synaptic input. Further studies are underway to elucidate how transcription and splicing of glutamate receptor genes are regulated after optic nerve lesions and to proove the physiological significance of this hitherto unknown aspect of post-lesion plasticity.

6. Immediate early gene response to optic nerve injury of RGC

The expression of immediate-early genes (IEG) after lesions of the central nervous system has been studied extensively in recent years. It is generally accepted that IEG encode transcription factors involved in the genomic response of cells to a variety of noxious stimuli. Especially the protooncogenes c-fos and c-jun have an established role in the brain mediating the genomic response to neuronal injury. Moreover, it has been hypothesized that c-jun expression precedes either neuronal death or a regenerating neuronal response [10,46] after brain lesions.

The time course of IEG expression has been studied in axotomized retinal ganglion cells shortly after the trauma and during neuronal regeneration [19,22,23]. Interestingly, it was found that axotomy leads to the transient but exclusive expression of c-Jun in the retinal ganglion cell layer [19,22,23,27], while neither c-fos nor other proteins of the jun-family were found to be upregulated. These studies emphasize that c-jun mRNA and protein are induced in surviving but disconnected RGC and it was hypothesized that c-jun expression precedes a sprouting response. The failure of axonal sprouting into the optic nerve is thereafter accompanied by the downregulation of c-Jun and subsequent cell death. Moreover, the accumulation of c-Jun immunoreactivity in apoptotic cells led other investigators to suggest that c-Jun expression is also involved in the initiation of programmed cell death after axotomy (for review see [20]).

We examined the expression of c-fos, fosB, c-jun, junB, junD, srf and pc4 mRNA after partial optic nerve crush in the adult rat retina by *in situ* hybridization. Optic nerve injury leads exclusively to the expression of c-jun while no other immediate early gene was upregulated after the lesion [31]. Within the retinal ganglion cell layer, cellular label representative for c-jun mRNA was found after two days, three days, one and two weeks post-injury. This expression pattern at the transcript level was largely in accordance with the appearance of c-Jun immunoreactivity in retinal flat mounts. Injection of an antisense but not a missense oligonucleotide against c-jun two and three days after crush resulted in less survival of connected RGCs as evidenced by retrograde labeling with horseradish peroxidase (HRP) [31]. Immunocytochemical stainings with a polyclonal antibody documented a specific attenuation of c-Jun immunoreactivity after injection of the antisense but not the missense oligonucleotide. C-Jun antibody staining in retinal whole mounts pre- or post-

labeled after crush by intratectal administration of fluorogold showed strong c-Jun immunoreactivity preferentially in connected RGCs and also in a population of axotomized RGC. Double labeling with an antibody directed against the transcription factor ATF-2 revealed strong co-expression of both c-Jun and ATF-2 exclusively in connected RGCs and not in axotomized cells [31]. Taken together, these data indicate that the coexpression of c-Jun with high but not with low levels of ATF-2 could be an important factor for the maintenance of axonal connections of surviving RGC after partial nerve injury. In axotomized RGC, however, low levels of ATF-2 and the coexpression of c-jun may be related to cell death. At present it is also unclear whether antisense knockdown after partial nerve injury leads exclusively to a disconnection of those RGC which otherwise remain connected with their target or to cell death of this population. The idea that cell survival requires high levels of ATF-2 and the simulataneous expression of c-Jun emphasizes a role for c-jun as a survival factor in those RGCs which remain connected with their target. This hypothesis has been suggested previously by Herdegen and colleagues [20]. In addition, the antisense knockdown experiments after partial nerve crush lead to the notion that c-Jun is an essential part of a signal transduction mechanism necessary for the stability or repair of axonal connections and/or cell survival. Thus, the connection of RGC to the SC seems to depend on an integrated cellular response to axonal injury in which c-Jun is indispensable.

7. Identification of differentially expressed genes after axonal injury

In an effort to study molecular mechanisms of postlesion plasticity we have also performed a screen for differentially expressed genes in the retina after diffuse axonal injury. One of the identified gene products significantly up-regulated in the retinal ganglion cell layer is the cell adhesion kinase-β (CAKβ) [29]. CAKβ/PYK2 and its closest homologue, pp125FAK, belongs to a family of cytoplasmic non-receptor tyrosine kinases, which are crucially involved in the formation of adhesion contacts. We could show by Northern blot analysis and RT-PCR that CAKβ/PYK2 transcript levels are clearly increased in the retina after nerve crush. Furthermore, *in situ* hybridization experiments with retinal cryostat sections revealed elevation of silver grain accumulation for CAKβ/PYK2 but not for pp125FAK between day 2 and two weeks postinjury. The increase in CAKβ/PYK2 immunoreactivity was restricted to the optic nerve, as evidenced by western blot analysis, and to axons of the RGC as shown by CAKβ/PYK2 immunostainings in retinal whole mounts. Increased CAKβ/PYK2 immunoreactivity was confined to the axoplasm of the optic nerve proximal to the retina and the crush site. These cellular compartments are damaged by diffuse mechanical shear stress, but they remain in contact with the soma, e.g. via anterograde axonal transport. We found that one putative substrate of CAKβ/PYK2, which is highly phosphorylated after crush, is the c-Jun amino-terminal kinase 1 (JNK1) but not JNK2. In addition protein levels of JNK1 but not JNK2 were elevated in the proximal optic nerve. As already outlined above, partial optic nerve injury leads to the induction of c-Jun in both axotomized RGC and in RGC which maintain connected with their target. Antisense knock-down of c-Jun significantly reduces the number of connected RGCs. Moreover, ATF-2, another substrate of JNK, is downregulated in axotomized RGCs but not in connected RGCs. Thus, multiple targets are conceivable for retrograde CAKβ/PYK2 and JNK signaling pathways to the nucleus after axonal injury.

This prompted us to investigate how CAKβ/PYK2 and its homologue, the focal adhesion kinase pp125FAK, are regulated in rat brain after fluid percussion injury. This is a commonly used model of closed head neurotrauma. Staining for pp125FAK in uninjured animals was observed especially in cortical layers 3 and 5 and in the hippocampus, with little or no signal in white matter tracts [5]. Fluid percussion injury leads to a downregulation of pp125FAK and CAKβ/PYK2 in cortical areas of the lesioned hemisphere but no change in immunoreactivity in the opposite uninjured cortex. In the hippocampus, the white matter, the fimbria and the cortex pp125FAK-immunoreactive cells with glial morphology were visible. CAKβ/PYK2 could be found in the uninjured brain especially in neuronal cells with staining of axonal fibers in white matter. Already one day after injury, the white matter tract immunoreactivity for CAKβ/PYK2 was remarkably enhanced. Axons were visible with differing diameters. Some of them appeared to be swollen, which is supposed to be a first sign for degeneration. Increased CAKβ/PYK2-immunoreactivity was observed for more than a week, whereas the neuronal staining in the injured hemisphere decreased, with no alterations in staining intensity in other brain areas. These data emphasize that both kinases are differentially regulated by neurotrauma. We are currently investigating whether the signaling pathways that are triggered by both enzymes are involved in processes leading to cell death/cell survival and reactive astroglia proliferation.

8. Retinofugal axonal projections after partial optic nerve crush

We also investigated the effects of retinal N-methyl-D-aspartat (NMDA)-toxicity and controlled optic nerve crush (ONC) on the retinofugal projection and its connectivity with the main targets of rat retinal axons, contralateral SC and dorsolateral geniculate (DLG) (Kreutz, Weise, Böckers and Sabel, unpublished observations). Using intravitreous injections of different anterogradely transported tracers (HRP, RITC, ^{125}IbFGF) we labeled the contralateral optic tract (OT), SC and DLG at various time points after lesion. We could show a recovery of tracer signal in the contralateral optic tract (OT), SC and DLG following an initial signal

loss after both excitotoxic and traumatic injury of RGC. While the topography of tracer signal after lesion was unaltered in the DLG, recovery of tracer signal in the SC was confined to its rostromedial part after both NMDA-toxicity and ONC. Moreover, rostromedial lateralisation of ^{125}IbFGF signal preceeded that of HRP. Retinal bFGF expression, however, was only slightly increased after ONC. The restriction of tracer accumulation was not due to a selective destruction of non dorso-temporal axonal fibers by the crush procedure since i.o. NMDA-injections led to comparable results. The altered topography of axonal transport can also not be explained by a selective degeneration of retinal ganglion cells projecting to those regions of the SC where loss of tracer signal was observed because neither retinal excitotoxicity induced by NMDA-injections [49,58] nor optic nerve crush [43] leads to a selective degeneration of RGC in certain quadrants of the retina. Taken together this evidence led us believe that the tracing experiments demonstrate a true change in the topography of retino-collicular axonal transport and not a change in the selective uptake or transport of tracer by a subpopulation of RGC in the dorso-temporal quadrant of the retina.

It has been shown by several groups that after optic nerve transection, retinal ganglion cells are able to regrow through transplanted peripheral nerves sutured to the transected nerve stump [36,56]. These regenerating axons are reportedly able to innervate the adult SC and to form functional and ultrastructurally normal synapses with target cells [44,52]. Most interestingly, it was found that the deafferented SC re-expresses axonal guidance that are responsible for the establishment of the retino-tectal topography [2,64]. Thus, the preference of neurite outgrowth in a stripe assay from membrane preparations of the adult deafferented SC was partially restored in that RGC axons from the temporal retina avoid growing on membrane stripes from the posterior SC whereas nasal retinal axons exhibited no growth preference [2,64]. The reexpression of attractive cues in the anterior SC after partial deafferentation would also partially explain why tracer signals accumulate preferentially in this region. But an important issue still raised by this study is why and by which mechanism is the topography of axonal transport altered in the adult rat. Experiments are therefore underway to clarify the functional consequences of these alterations and the mechanisms that are responsible for this striking plasticity of the adult rat visual system.

9. Conclusions

The crush of the optic nerve has proven to be particularly useful for the study of molecular plasticity at the cellular level under conditions of partial axonal injury. A summary of our findings utilizing the partial crush model is provided in Fig. 2. With this *in vivo* model, it should be possible to gain a better understanding of signaling pathways from the injured axonal compartment to the nucleus. Moreover, the study of these signaling pathways will be helpful to discern the molecular machinery involved in either cell death or survival in response to diffuse axonal injury, a type of brain lesion that is commonly underestimated in its significance of secondary degeneration after closed head neurotrauma.

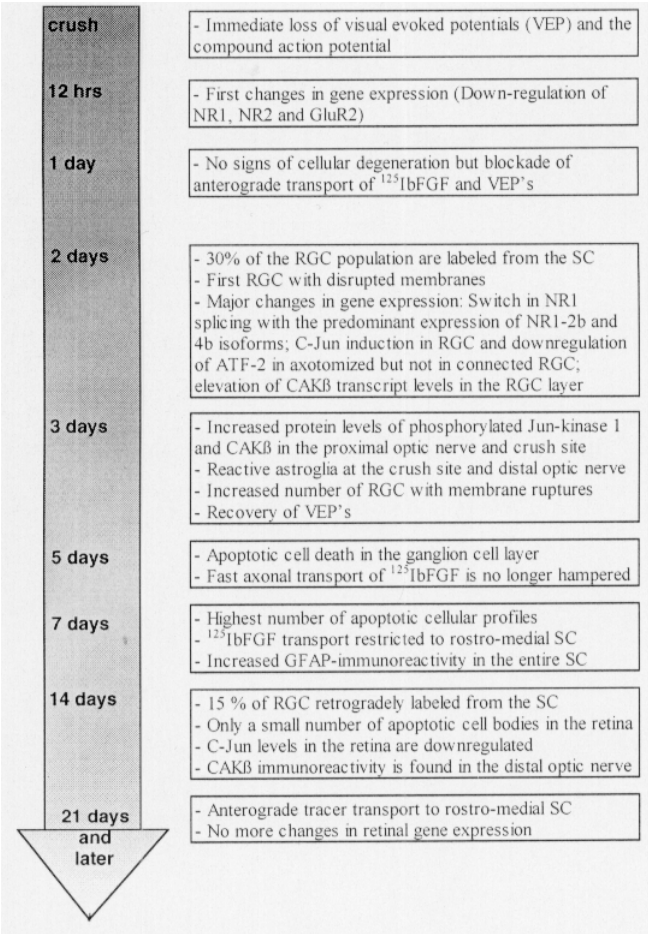

Fig. 2. The response of RGC to partial optic nerve injury that we found in our studies is summarized in this cartoon. A flow chart describes the major findings in their temporal occurance. First changes in gene expression after ONC are found 12 hours post-injury. Interestingly, transcriptional regulation of gene expression is most prominent two days post-crush, coinciding in time with first signs of cellular degeneration but preceeding massive cell death. The major period of molecular plasticity in response to partial optic nerve crush injury is terminated after the second and third week. Thereafter, little or no changes were found in gene expression, axonal transport and other physiological parameters. Only those time points are shown when changes were first detected, which does not necessarily imply that they first occur at this time point. Moreover, causal relations between the different molecular

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